# Spatially Restricted Expression of a Member of a New Family of Murine *Distal-less* Homeobox Genes in the Developing Forebrain

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**The homeobox genes of *Drosophila* perform key functions in embryonic pattern formation, and their vertebrate counterparts may play similar developmental roles. Using polymerase chain reaction technology, we have identified four murine homologs of the *Drosophila Distal-less* homeobox gene that are expressed in midgestation embryos. The homeodomains encoded by these genes vary considerably from other known homeodomain sequences and represent a new family of vertebrate homeobox genes. We isolated a cDNA for one of these genes (*Dlx*-2) and studied its expression by in situ hybridization from 8.5 days postcoitum (pc) until postnatal day 1. *Dlx*-2 shows a restricted pattern of expression in the ventral forebrain, extending from the olfactory bulb to the ventral diencephalon. This domain of expression may delineate an ontogenetically defined subdivision within the forebrain. The murine *Distal-less* genes are the first homeobox genes described whose expression in the central nervous system is exclusively restricted to the forebrain. Thus, the *Distal-less* genes may contribute missing positional cues not provided by the previously identified vertebrate homeobox genes.**

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In *Drosophila*, it is well established that developmental control genes act in a hierarchical manner in the regulation of development (for reviews see Ingham, 1988; Akam, 1989). Genetic analyses have shown that the basic body plan is established through gradients of maternally encoded proteins that regulate the expression of zygotic segmentation genes. Through a series of threshold responses, the segmentation genes define regionally distinct compartments whose identity is specified by subordinate homeotic genes (Gehring, 1987). Many of these *Drosophila* regulatory genes are conserved in evolution, and homologs have been identified in other organisms, including vertebrates. The high degree of conservation among such genes suggests that some of the regulatory functions are maintained as well (Dressler and Gruss, 1988).

Many developmental control genes encode transcription factors and contain one of several DNA binding motifs (for a review see Dressler, 1989). One of these motifs is the homeobox, which encodes a 61–amino acid DNA-binding domain that was first identified in the homeotic genes of *Drosophila* (for a review see Gehring, 1987). On the basis of their deduced amino acid sequence, these homeobox genes have been subgrouped into several different classes (Scott et al.,1989). The majority of murine homeobox genes identified to date, the so-called *Hox* genes, contain a homeobox that is homologous to the *Drosophila Antennapedia* gene. *Hox* gene family members are arranged in four chromosomal clusters, and are expressed in overlapping domains of expression in the central nervous system (CNS) and somitic mesoderm. The most anterior boundary of their expression lies in the hindbrain. They are expressed transiently during midgestation and are thought to confer positional identity along the axial skeleton and CNS (Holland and Hogan, 1988; Graham et al., 1989; Kessel and Gruss, 1990).

The *Distal-less* (*Dll*) homeobox gene of *Drosophila* is expressed during cellularization of the blastoderm in a group of cells that give rise to the embryonic limbs (Cohen et al., 1989; Cohen, 1990). The expression of *Dll* serves as a molecular marker for cells of limb primordia in the embryo and imaginal discs. Genetic analysis showed that the development of thoracic legs and the peripheral embryonic sensory organs in the head depends on the *Dll* gene product (Cohen and

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Dll                KPRTIYSSLQLQQLNRRFQRTQYLALPERAELAASLGLTQTQVKIWFQN
Dll A (Dlx-2)           F  AA Q    KT              S
Dll B (Dlx-1)           L  QA N    QT              S
Dll C (Dlx-3)           Y  LA Q    KA              Q
Dll D (Dlx-4)           L  AA Q    KA              S
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**Figure 1. Comparison of murine Distal-less–like homeodomain sequences.**

The predicted amino acid sequence of the *Drosophila Dll* gene and the four murine sequences amplified by PCR are shown. The names of the corresponding murine genes are given in parentheses (see text). Bold letters indicate the variable residues. The underlined sequences indicate the regions used to make degenerate primers for PCR. The single letter code for amino acid residues is as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gln; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Glu; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

Jürgens, 1989). The homeodomain of *Dll* is quite divergent and distinct from that of other homeobox genes (Cohen et al., 1989).

The involvement of the *Drosophila Dll* gene in specific developmental pathways and the conservation of homeobox genes in vertebrates led us to search for a murine homolog. Using polymerase chain reaction (PCR)-based techniques, we have shown that at least four genes containing a *Dll*-like homeobox are expressed in midgestation embryos, and have isolated a cDNA clone for one of these genes. In situ hybridization analysis has shown that this gene is expressed in a highly restricted pattern in the developing forebrain of the mouse embryo. The expression pattern is unique among previously described homeobox genes whose expression is limited to more caudal regions of the CNS. Our data suggest that this murine *Distal-less* homolog plays a specific role in the development of the forebrain.

## RESULTS AND DISCUSSION

### *Identification of the* Distal-less *Homeobox Gene Family in the Mouse*

To determine whether sequences homologous to the *Drosophila Distal-less* (*Dll*) homeobox were present in the mouse and expressed during development, we synthesized fully degenerate primers corresponding to codons from both the $NH_2$-terminus domain outside the helix 1 region and the highly conserved COOH-terminus of helix 3 of the *Dll* homeodomain (Cohen et al., 1989) (Fig. 1). These primers were used in amplification reactions with 13.5-day postcoitum (pc) embryonic mouse cDNA as template. PCR products of the expected size were cloned and sequenced. Four different homeobox sequences were identified, whose predicted amino acid sequences showed homology to the *Drosophila Dll* homeodomain (Fig. 1). The murine *Dll* sequences are extremely conserved among themselves and show a similar degree of conservation when compared to the *Drosophila* sequence. The amplified regions show between 81% and 94% homology on the amino acid (aa) level to the *Drosophila Dll* homeodomain and between 83% and 94% homology to each other. The actual homology is probably greater if one considers that the compared regions do not include most of helix 3, the most conserved region of the homeodomain sequence.

Variations in aa residues are not randomly distributed along the 37-aa amplified region, but occur at six distinct positions. The *Drosophila* sequence varies at an additional position (residue 15). With the exception of residue 37, all of the variable positions are located in helix 1 and the hinge region between helix 1 and helix 2. Five of the changes in the aa sequences are conservative. The only changes resulting in an alteration of charge are a lysine to tyrosine or phenylalanine change at the second position of helix 1, and an arginine to glutamine change at residue 22 downstream from helix 1. Among the four amplified sequences, a much lower degree of conservation is seen at the nucleotide level with only 8 of the 37 codons being identical (data not shown). Two of the four sequences (Dll A and Dll B) were cloned repeatedly, indicating that they might be more highly expressed than the other two.

These results indicate the existence of an extremely conserved family of genes containing *Distal-less* homeobox sequences in the mouse. We have thus far isolated cDNA clones corresponding to two of the amplified homeobox sequences (Dll A and C) from embryonic cDNA libraries (see below and unpublished results). The Dll A sequence and its expression pattern are described in this manuscript. Recently, the isolation of *Dlx*, a gene containing our Dll B homeobox sequence, has been described (Price et al., 1991). We concur that the *Dlx* nomenclature be adopted for this gene family, and suggest that the *Dlx* gene of Price et al. be renamed *Dlx*-1, and the Dll A homeobox gene

**Figure 2. cDNA sequence and deduced amino acid sequence of *Dlx*-2.**

(**A**) The homeobox sequence is double underlined and the two glycine/serine regions are underlined. (**B**) Comparison of the *Dlx*-2 homeodomain with other classes of *Drosophila* homeodomain sequences. Boxes show the three helices. Invariant residues are shaded. Sources for sequences are as follows: *Dll* (Cohen at al., 1989), *Antp* (McGinnis et al., 1984), *en* (Poole et al., 1985), *prd* (Frigerio et al., 1986), *eve* (Macdonald et al., 1986), NK1 (Kim and Nirenberg, 1989).

**A**

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1    CACCAGCAGCCCCCGAGCGGTGCGGGCGCCGGCCCTGGCGGCAACAGCAACAGCAGCAGC   60
1    H  Q  Q  P  P  S  G  A  G  A  G  P  G  G  N  S  N  S  S  S    20

61   AGCAACAGCAGCCTGCACAAGCCCCAGGAGTCGCCAACCCTCCCGGTGTCCACGGCTACG  120
21   S  N  S  S  L  H  K  P  Q  E  S  P  T  L  P  V  S  T  A  T    40

121  GACAGCAGCTACTACACCAACCAGCAGCACCCGGCGGGCGGCGGCGGCGGGGGGGCCTCG  180
41   D  S  S  Y  Y  T  N  Q  Q  H  P  A  G  G  G  G  G  G  A  S    60

181  CCCTACGCGCACATGGGCTCCTACCAGTACCACGCCAGCGGCCTCAACAACGTCTCCTAC  240
61   P  Y  A  H  M  G  S  Y  Q  Y  H  A  S  G  L  N  N  V  S  Y    80

241  TCCGCCAAAAGCAGCTACGACCTGGGCTACACCGCCGCGTACACCTCCTACGCGCCCTAC  300
81   S  A  K  S  S  Y  D  L  G  Y  T  A  A  Y  T  S  Y  A  P  Y   100

301  GGCACCAGTTCGTCCCCGGTCAACAACGAGCCGGACAAGGAAGACCTTGAGCCTGAAATC  360
101  G  T  S  S  S  P  V  N  N  E  P  D  K  E  D  L  E  P  E  I   120

361  CGAATAGTGAACGGGAAGCCAAAGAAAGTCCGGAAACCACGCACCATCTACTCCAGTTTC  420
121  R  I  V  N  G  K  P  K  K  V  R  K  P  R  T  I  Y  S  S  F   140

421  CAGCTGGCGGCCCTTCAACGACGCTTCCAGAAGACCCAGTATCTGGCCCTGCCAGAGCGA  480
141  Q  L  A  A  L  Q  R  R  F  Q  K  T  Q  Y  L  A  L  P  E  R   160

481  GCCGAGCTGGCGGCGTCCCTGGGCCTCACCCAAACTCAGGTCAAAATCTGGTTCCAGAAC  540
161  A  E  L  A  A  S  L  G  L  T  Q  T  Q  V  K  I  W  F  Q  N   180

541  CGCCGATCCAAGTTCAAGAAGATGTGGAAAAGCGGCGAGATACCCACCGAGCAGCACCCT  600
181  R  R  S  K  F  K  K  M  W  K  S  G  E  I  P  T  E  Q  H  P   200

601  GGAGCCAGCGCTTCTCCTCCTTGTGCCTCCCCGCCGGTCTCGGCGCCAGCATCCTGGGAC  660
201  G  A  S  A  S  P  P  C  A  S  P  P  V  S  A  P  A  S  W  D   220

661  TTCGGCGCGCCGCAGCGGATGGCTGGCGGCGGCCCGGGCAGCGGAGGCGGCGGTGCGGGC  720
221  F  G  A  P  Q  R  M  A  G  G  G  P  G  S  G  G  G  G  A  G   240

721  AGCTCTGGCTCCAGCCCGAGCAGCGCCGCCTCGGCCTTTCTGGGAAACTACCCGTGGTAC  780
241  S  S  G  S  S  P  S  S  A  A  S  A  F  L  G  N  Y  P  W  Y   260

781  CACCAGGCTTCGGGCTCCGCTTCACACCTGCAGGCCACAGCGCCACTTCTGCATCCTTCG  840
261  H  Q  A  S  G  S  A  S  H  L  Q  A  T  A  P  L  L  H  P  S   280

841  CAGACTCCGCAGGCGCACCATCACCACCATCACCACCACCACGCAGGCGGGGGCGCCCCG  900
281  Q  T  P  Q  A  H  H  H  H  H  H  H  H  H  H  A  G  G  G  A  P   300

901  GTGAGCGCGGGGACGATTTTCTAACCTCGAGTGGACAGCGTCTGAGACTTGAGAACTGAG  960
301  V  S  A  G  T  I  F  *                                        320

961  ACACCGTGTTCTTCAGCACGCGTCCTGTAGCTAGAGGCTCGAACCTACCAGCCCGCGACC 1020

1021 GCCACCCACCCAGCGGTATTGGAAGTGGCGACCAGGGGTGACCGGTCGTGGAGCCACTTC 1080

1081 TCGCACTGCCACGGAGCAGCCGGAAACAAGGAGGCCTCGTGGGATCCTTTAGTGCCAAGC 1140

1141 CTGGCGTCCCTGGCCTCAATTCCGACCTGAGCCCCGGCCACTTTTAGGCCATCCTCGGAC 1200

1201 TTGTCGCCCCTTCCCAGCCCCCTCAGCGTGATCCTCAGGGTCCTTGGTCTCTTCAGGCCT 1260

1261 GAGCGGGGTCAGGCACTGGGACGCCTTCCCCGCCCTCGAGGCTTCCCGGCTCTCAGAGGC 1320

1321 TCCTTTTTTCTCTCCTGCTCCAGGCCTCAGCTCCAGTCCGCTTCGGTTATTGACCTCCTG 1380

1381 GGTCCTACTCCACCCTATCACCTACCCCCGTGACCCCCGGGATCCCGCCGGTTTTTTCCC 1440

1441 CTCCTGGGGGCTCATGGGGGA 1461
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**B**

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Dlx-2  VRKPRTIYS SFQLAALQRRFQ KTQYLA LPERAELAASL GLT QTQVKIWFQNR RSKFKKMWK
Dll    MRKPRTIYS SLQLQQLNRRFQ RTQYLA LPERAELAASL GLT QTQVKIWFQNR RSKYKKMMK
Antp   RKRGRQTYT RYQTLELEKEFH FNRYLT RRRRIEIAHAL CLT ERQIKIWFQNR RMKWKKENK
en     EKRPRTAFS SEQLARLKREFN ENRYLT ERRRQQLSSEL GLN EAQIKIWFQNK RAKIKKSTG
prd    QRRCRTTFS ASQLDELERAFE RTQYPD IYTREELAQRT NLT EARIQVWFSNR RARLRKQHT
eve    VRRYRTAFT RDQLGRLEKEFY KENYVS RPRRCELAAQL NLP ESTIKVWFQNR RMKDKRQRI
NK1    PRRARTAFT YEQLVSLENKFK TTRYLS VCERLNLALSL SLT ETQVKIWFQNR RTKWKKQNP
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described in this report *Dlx*-2. A cDNA containing a sequence encoding the Dll D homeobox has not yet been isolated. However, because the Dll D nucleotide sequence is quite distinct from the other Dll homeobox sequences (data not shown), we believe it is derived from a bona fide gene and not a PCR artifact. Thus, taken together, our data indicate that the mouse genome contains at least four *Distal-less* like genes that are expressed in midgestation embryos.

### *Isolation and Characterization of a* Dlx-*2 cDNA Clone*

By screening an 11.5-day embryonic cDNA library with a mixture of the four amplified Dll homeobox sequences, a 1.4-kb cDNA clone was isolated. The nucleotide sequence of this clone was determined and is shown in Fig. 2A along with the predicted aa sequence. It contains a homeobox sequence identical to that of Dll A, and is designated *Dlx*-2. This sequence is not full length, as it does not contain an initiating ATG codon and lacks a polyadenylation signal at the 3′ end.

Comparison of representative homeodomain sequences from several classes of homeobox genes with that of *Dlx*-2 illustrates that the *Distal-less* homeobox gene family is quite unique (Fig. 2B). Although strongly homologous to the *Drosophila Dll* homeodomain (53/61 residues), the homeodomain of *Dlx*-2 is only weakly related to other sequences. The extent of homology between *Dlx*-2 and *Dll* outside of the homeodomain is not known since the full sequence of the *Drosophila* gene has not been published. *Dlx*-2 contains all the conserved invariant residues in the homeodomain, but shows considerable variation at other positions. The predicted protein sequence of *Dlx*-2 contains a 117-aa long COOH terminus downstream of the homeodomain, in contrast to many homeobox genes that have the homeodomain close to the COOH terminus. The predicted $NH_2$-terminal and COOH-terminal portions of *Dlx*-2 each contain a glycine/serine rich region. Furthermore, there is a stretch of 9 histidine residues near the COOH terminus (Fig. 2A). The significance of this highly charged region is not known.

### *Expression of* Dlx-*2 in the Developing Forebrain*

The developmental profile of *Dlx*-2 expression was determined by Northern blot analysis of RNA isolated from microdissected embryos. The *Dlx*-2 cDNA probe hybridizes to a 2.6-kb message in RNA from heads but not from trunks of 10.5- to 15.5-day embryos (Fig. 3). A survey of different tissues from 18.5-day embryos shows expression of *Dlx*-2 in the brain only. We were unable to detect *Dlx*-2 transcripts in RNA from 18.5-day embryonic testis, stomach, spleen, heart, liver, skin, kidney, and lung and adult brain (data not shown).

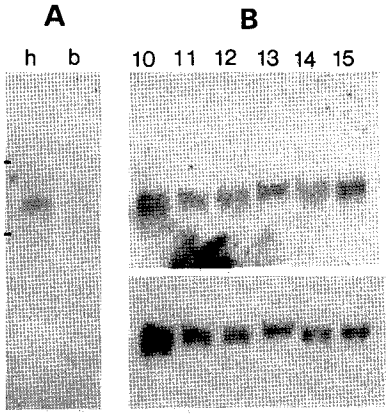


**Figure 3. Detection of *Dlx*-2 by Northern blot analysis.**

(**A**) Total RNA (30 μg) prepared from 11.5-day pc embryonic heads (h) and bodies (b) was blotted and hybridized to *Dlx*-2 probe. (**B**) Upper panel: 40 μg of RNA from heads of 10.5- to 15.5-day embryos. Lower panel shows hybridization to glyceraldehyde-3-phosphate-dehydrogenase (Fort et al., 1985) as a control for integrity and amount of RNA. Marks on (A) indicate positions of 28S and 18S rRNA.

In situ hybridization to tissue sections of embryonic and newborn mice (from 8.5 days pc through postnatal day 1) was utilized to determine the spatial and temporal pattern of *Dlx*-2 expression. *Dlx*-2 transcripts were not observed at day 8.5 pc, but were detectable by 11.5 days pc. Consistent with the results from Northern blot analysis, specific hybridization was exclusively confined to the head. Moreover, *Dlx*-2 expression was restricted to discrete regions of the developing forebrain (Fig. 4). Within these regions, transcripts were localized to neuroepithelial zones in cells of the outer germinal or synthetic layer but not in cells of the proliferative zone directly adjacent to the ventricle (Altman and Bayer, 1986). Specifically, a strong signal was detected in regions of the basal telencephalon and ventral diencephalon. All labeled areas within the diencephalon are rudiments of hypothalamic structures (i.e., anterior and posterior hypothalamus and preoptic area) as described by Altman and Bayer (1986). Interestingly, the structures expressing *Dlx*-2 at this embryonic stage are derived from the basal plate.

By day 13.5 pc expression of *Dlx*-2 was seen in several discrete regions. Within the telencephalon, expression remained strong within the neuroepithelium of the basal telencephalon (Fig. 5, A to D). The boundaries of expression, particularly with respect to the dorsal limit, were very distinct. No transcripts were detected within the neuroepithelium of the cortex or hippocampus. Although the signal was still strongest within the synthetic zone, weaker labeling was also observed in the differentiating zone. At the telencephalic/diencephalic border, *Dlx*-2 was expressed in the neuroepithelium of the diagonal band of Broca (Fig. 5,

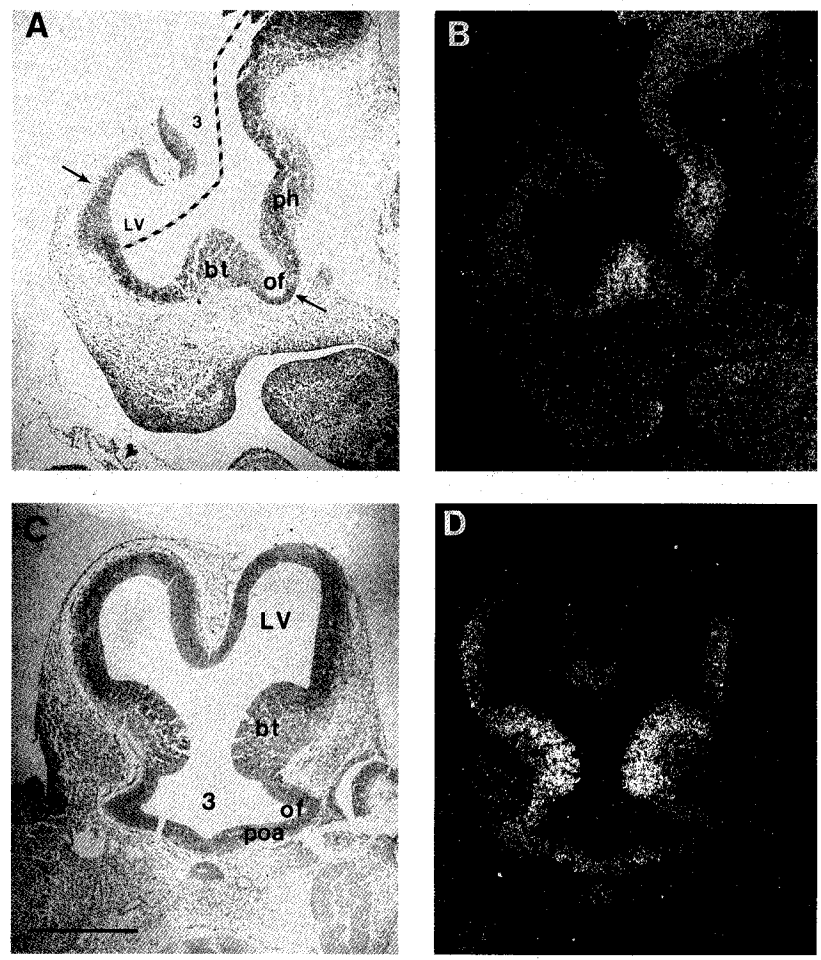


**Figure 4. Localization of *Dlx*-2 expression in 11.5-day embryos.**

(**A** and **B**) Sagittal section hybridized in situ to *Dlx*-2 antisense probe. Labeling is seen in the basal telencephalon neuroepithelium (bt) and the posterior hypothalamus (ph). The dotted line marks the border between the surface plate and the basal plate of the neuroepithelium. Arrows indicate the plane of section C. (**C** and **D**) Transverse section. *Dlx*-2 is expressed in the neuroepithelia of the basal telencephalon and preoptic area (poa). Bright field (A,C) and corresponding dark field (B,D). LV, lateral ventricle; of, optic fissure; 3, third ventricle. Scale bar=625μm.

C and D). Labeling continued into the diencephalon proper with cells in the preoptic region showing a strong signal. Weaker, but consistent, labeling was seen in cells in the anterior hypothalamus, lateral hypothalamus, and presumptive zona incerta (Fig. 5, E and F). Sharp expression domains were evident in the diencephalon, with the dorsal limit corresponding to the junction of the basal and alar plates, the sulcus limitans (Altman and Bayer, 1986).

As in all labeled areas mentioned, the synthetic and differentiating zones of the neuroepithelium showed stronger expression than cells in the proliferative zone. Consistent with the earlier expression patterns, all forebrain structures labeled at this stage are derived from the basal plate (Altman and Bayer, 1986).

At day 16.5 pc, the striking regional localization of *Dlx*-2 transcripts was similar to that seen at day 13.5, but more anatomically discernable structures could now be visualized. *Dlx*-2 expression was seen in the ventrolateral neuroepithelium of the olfactory lobes (Fig. 6, A and B). This labeling was continuous with cells showing a strong signal in the basal telencephalic area. At this stage the region directly ventrolateral to the lateral ventricle can be easily discernable as the developing striatum, that is the caudate/putamen neuroepithelium (Fig. 6, C to H). These cells maintain robust expression of *Dlx*-2 throughout embryonic development. By day 16.5, intense labeling of the synthetic and differentiating zones of the septal neuroepithelium was also visible (Fig. 6, E to H). However, the cortex and developing hippocampal regions remained unlabeled. Thus, a clear demarcation of *Dlx*-2 expression can be seen between the ventral (bottom two thirds) region of the neuroepithelium surrounding the lateral ventricles and the dorsal (top one third) region. *Dlx*-2 expression was still detected at the telencephalic/diencephalic border in the neuroepithelial zones of the diagonal band of Broca (data not shown). This labeling was continuous caudally, with cells expressing *Dlx*-2 in the neuroepithelial zones at the level of medial preoptic area and anterior hypothalamus. In this region, cells expressing *Dlx*-2 were not evenly dispersed along the third ventricle, but occurred in waves (Fig. 6, G and H). It is interesting to note that at this stage, the suprachiasmatic nucleus has formed and can be seen impinging on the ventral aspect of the third ventricle (Fig. 6, G and H). *Dlx*-2 expression is clearly absent within this nucleus. Further caudally within the diencephalon,

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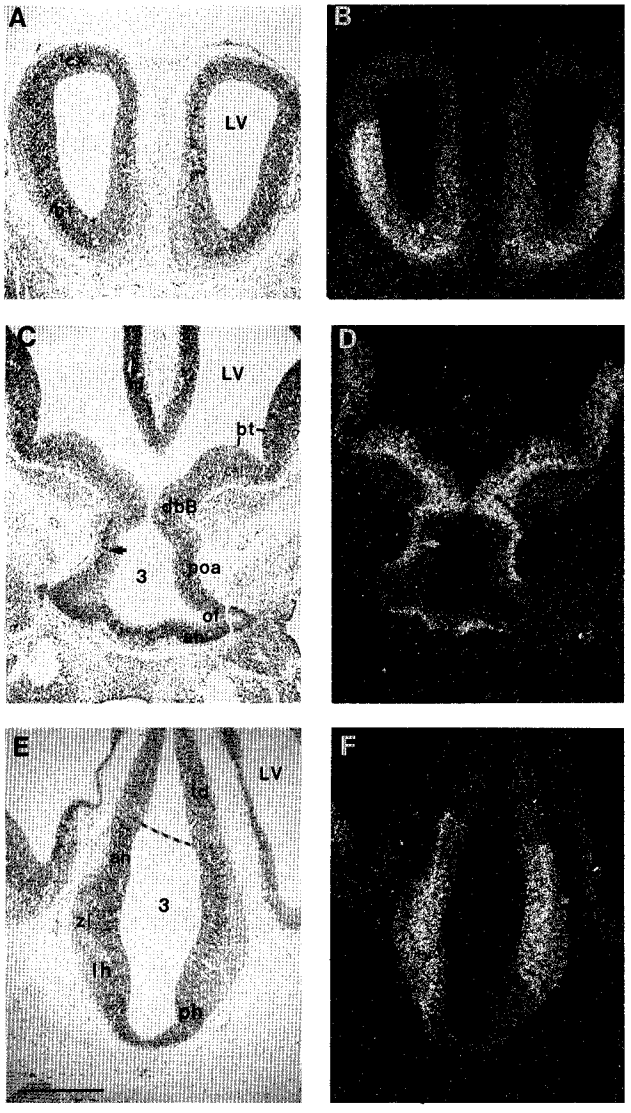


**Figure 5.** ***Dlx*-2 expression in telencephalon and diencephalon of 13.5-day embryos.**

Coronal sections in rostro-caudal sequence. (**A** and **B**) Section through rostral forebrain. *Dlx*-2 transcripts are seen in the basal telencephalon neuroepithelium (bt). Note the absence of transcripts in the cortical neuroepithelium (cx). (**C** and **D**) Section at the telencephalic/diencephalic junction at the level of the foramen of Monro. Strong labeling occurs in the basal telencephalon (bt) and the diagonal band of Broca (dbB). In the hypothalamic preoptic area (poa) and the anterior hypothalamus (ah) only the cells of the synthetic zone are labeled. Arrows indicate the zone of expression. No transcripts are seen in the optic fissure (of). (**E** and **F**) *Dlx*-2 expression is restricted to a region of the anterior hypothalamus (ah), the zona incerta (zi), and the lateral hypothalamus (lh) in the diencephalon. Note striking boundary of expression corresponding to the border between the alar (surface) plate and basal plate (sulcus limitans) (dotted line). Panels A,C,E are bright field; panels B,D,F are dark field. hi, hippocampus neuroepithelium; LV, lateral ventricle; 3, third ventricle; ph, posterior hypothalamus; td, dorsal thalamus. Scale bar=500 μm.

weak labeling was seen in the region of the paraventricular nucleus and zona incerta (data not shown), whereas robust labeling occurred within cells of the arcuate nucleus (Fig. 6, I and J). These three diencephalic nuclei are the only examples within the forebrain of differentiated anatomical structures that express *Dlx*-2, all other labeling being confined to neuroepithelial zones.

At 18.5 day pc and postnatal day 1, the location of cells expressing *Dlx*-2 in the forebrain was similar to that seen at day 16.5 pc. However, the expression of *Dlx*-2 is downregulated, such that by birth the intensity of the hybridization signal had significantly decreased. This was most evident in the neuroepithelial cells of the caudate/putamen, septal and olfactory areas (Fig. 7), where until day 16.5 pc, the signal was the strongest. Labeling throughout the diencephalon, including the paraventricular and arcuate nucleus, was extremely low or undetectable at birth (data not shown).

### Dlx-*2 Expression in the Developing Tooth*

In addition to labeling of ventral forebrain regions, *Dlx*-2 transcripts were detected at 13.5 days pc in the epithelium of the tooth germ (Rugh, 1968; data not shown). This labeling became increasingly stronger in later developmental stages. By 16.5 days, labeling was localized in the upper and lower incisor anlagen, with the signal confined to the ameloblasts (Fig. 8, A and B). Unlike the signal in the forebrain, the labeling in the teeth was still strong at birth and now occurred in the developing molars as well as the incisors (Fig. 8, C and D).

Organogenesis of the tooth occurs through inductive interactions between oral ectoderm and neural crest–derived mesenchyme. These components ultimately give rise to the enamel producing ameloblasts and the dentine producing odontoblasts, respectively (for a review see Thesleff and Hurmerinta, 1981). The development of the tooth is one of the most elegant examples of reciprocal epithelial-mesenchyme interactions in organogenesis, with evidence that both neural crest and epithelial components convey spatial information (i.e., specification, shape, and patterning of the teeth) (Lumsden, 1988). Interestingly, the homeobox gene *Hox* 7.1 is expressed in a complementary pattern in the mesenchyme immediately surrounding the developing tooth bud (MacKenzie et al, 1991). Since it is possible to manipulate this system experimentally in tissue explants and transplantations (Kollar and Baird, 1969; Lumsden, 1988), the roles of these two homeobox genes, *Dlx*-2 and *Hox* 7.1, in the induction and differentiation of this organ may be examined.

### *Expression Domain of* Dlx-*2 Gene*

The expression of *Dlx*-2 is spatially restricted to a distinct region of the basal telencephalon and ventral diencephalon. Precise boundaries of expression are the hallmarks of homeobox gene expression, and support the widely held theory that these genes convey spatial information in developing systems. The most striking boundary of *Dlx*-2 expression in the forebrain is the

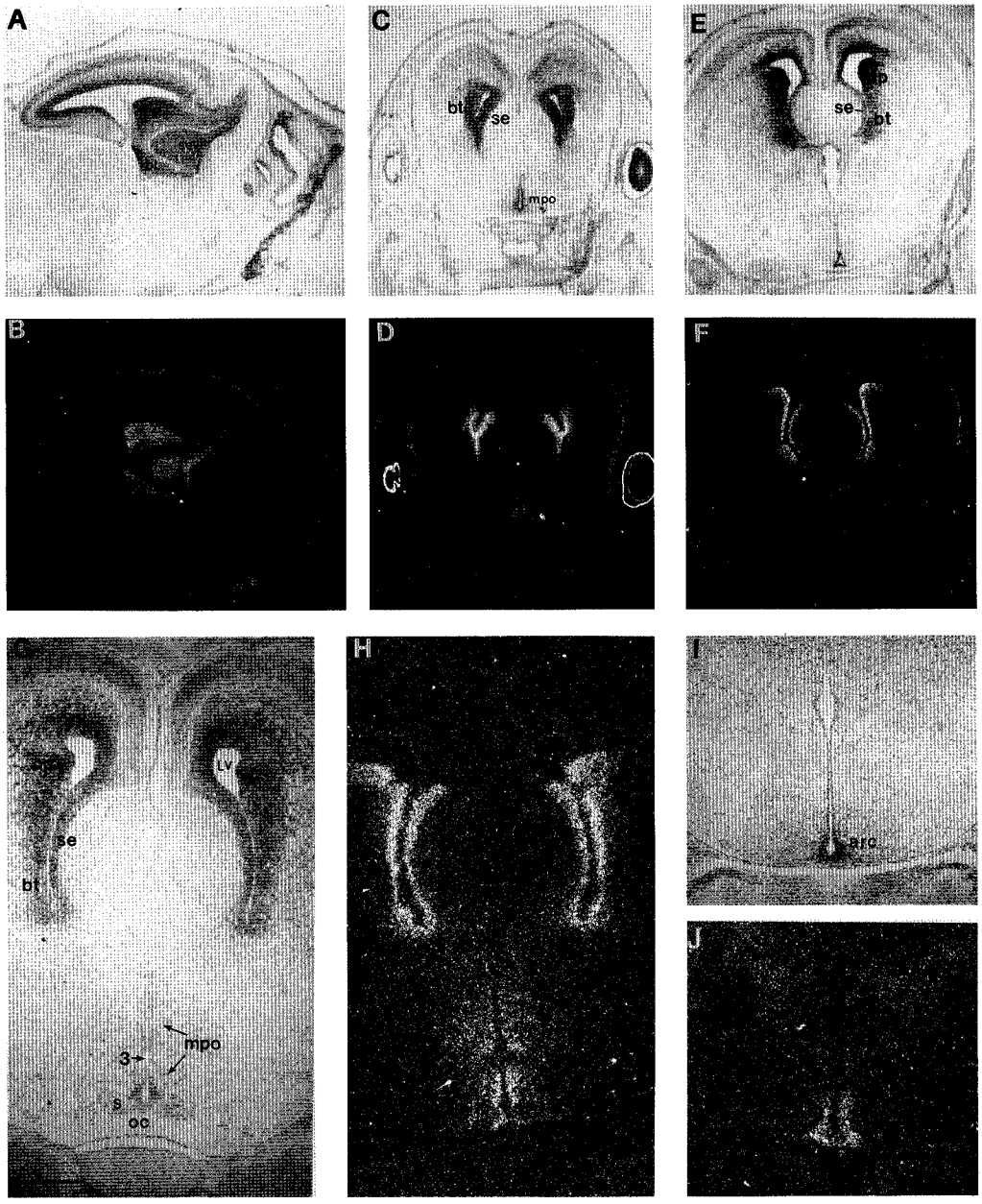


**Figure 6.** ***Dlx*-2 expression in 16.5-day pc embryos.**

In situ hybridization of a parasagittal section (**A** and **B**) showing high expression of *Dlx*-2 in the neuroepithelium of the caudate/putamen (cp) and septal neuroepithelium (se). Labeling extends into the ventral region of the olfactory bulb (ob). (**C** and **J**) Coronal sections in rostro-caudal sequence. *Dlx*-2 transcripts are seen in the neuroepithelia of the septum (se) and the basal telencephalon (bt). In the hypothalamus, cells in the medial preoptic area (mpo) are labeled (**C** and **D**). More caudally, strong labeling is also seen in the caudate/putamen (cp) neuroepithelium but not in the cerebral cortex (cx). The apparent signal in the developing retina is due to the diffraction by pigment granules. (**G** and **H**) Higher magnification of a section lying between C and E. Note labeling in the medial preoptic nucleus (mpo) whereas the suprachiasmatic nucleus (s) shows only background labeling. The arcuate nucleus (arc) also expresses *Dlx*-2 (**I** and **J**). Labeling is seen in the subventricular (synthetic) region of the neuroepithelium and not in the cells lining the ventricle (proliferative zone). Panels A,C,E,G,I are bright field; panels B,D,F,H,J are dark field. Scale bar=1mm in panels A to F and 500 μm in panels G to J.

division between the alar and basal plates (sulcus limitans). *Dlx*-2–expressing cells are never seen across this border, and this observation supports the concept that the basal plate is a distinct ontogenetic entity (as discussed in Puelles et al., 1987).

We have based our interpretation of *Dlx*-2 expression on the evolution of the labeling pattern from early stages (11.5 to 13.5 days pc) to later in development, as the brain differentiates into specific anatomically discernible structures. A number of classification systems

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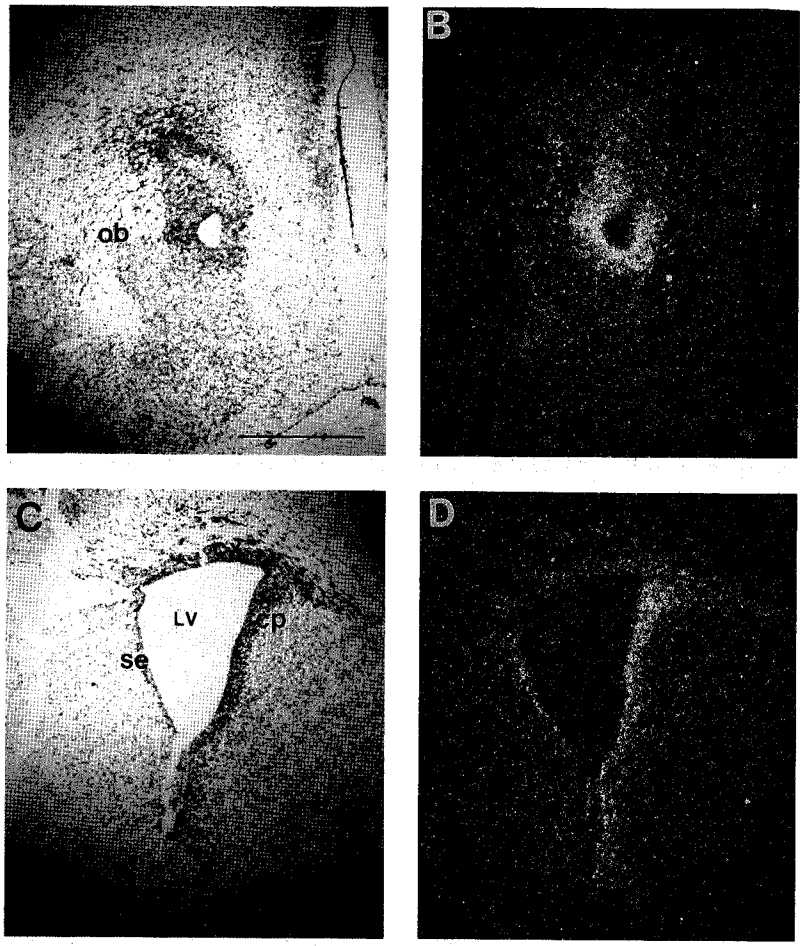


**Figure 7. *Dlx*-2 expression in newborn mice.**

Coronal section through the olfactory bulb (ob) (**A** and **B**). As in earlier stages, *Dlx*-2 expression is seen in the ventrolateral neuroepithelial cells of the synthetic zone. Note the absence of labeling in the dorsal part. (**C** and **D**) Labeling in the telencephalon is greatly reduced with only the neuroepithelium of the caudate/putamen (cp) expressing moderate levels of *Dlx*-2. Panels A and C are bright field; panels B and D are dark field. cx, cortex; LV, lateral ventricle; se, septum. Scale bar=625 μm.

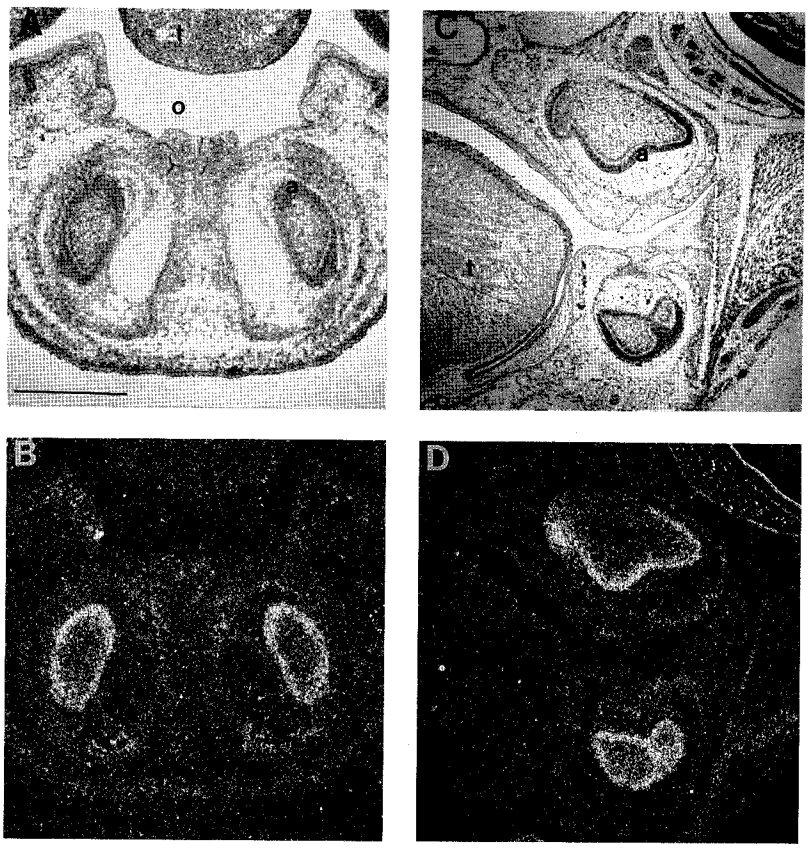


**Figure 8. Expression of *Dlx*-2 in tooth rudiments.**

Sections through 16.5-day pc incisors (**A** and **B**) and newborn molars (**C** and **D**). *Dlx*-2 expression is seen in the ameloblast layer (a). o oral cavity, t tongue. Scale bar=625 μm.

exist for the subdivision of the diencephalon into longitudinal tiers during development. We have used the system of Altman and Bayer (1986), founded on the work of His (1893), in our interpretation because it is based on ontogenetical relationships instead of on structural/functional areas in the adult. According to this system, all structures lying beneath the sulcus limitans or sulcus diencephalicus medius (Herrick, 1910) belong to the hypothalamus (not ventral thalamus and hypothalamus). The expression pattern of *Dlx*-2 supports this classification scheme since the labeling pattern is consistently within the ventral forebrain throughout development, and only one area, the zona incerta, would be classified by others as ventral thalamus.

Within its domain of expression, *Dlx*-2 is expressed in sub-germinal and differentiating neuroepithelia, but also in a few differentiated neurological structures (such as zona incerta, paraventricular and arcuate nuclei). However, this observation does not imply that *Dlx*-2–expressing nuclei are terminally differentiated. In fact, the paraventricular nucleus, which is temporally generated between 12 and 15 days of development (Altman and Bayer, 1986), has a subpopulation of neurons that are still maturing in newborn animals (Alstein and Gainer, 1988), at a time when *Dlx*-2 expression is strongly downregulated or absent. In general, *Dlx*-2 expression occurs in differentiating, premigrating cells; therefore, *Dlx*-2 may be involved directly in the differentiation process or in providing positional cues during differentiation.

### *Comparative Expression of the* Dlx *Gene Family in the Forebrain*

The other member of the *Dlx* gene family described to date, *Dlx* (*Dlx*-1), is also expressed in the basal telencephalon and ventral diencephalon of the developing forebrain (Price et al., 1991). The domain of expression of *Dlx*-1 overlaps extensively with that of *Dlx*-2, and comparative transcript mapping on serial sections with both probes will be necessary to accurately determine their respective boundaries. However, taking into account the published information, it would appear that these genes have distinct expression domains, particularly with respect to the rostro-caudal limits of expression. Caudally, expression of *Dlx*-1 is not detected past the optic chiasma (Price et al., 1991), whereas the posterior boundary of *Dlx*-2 expression extends into the more caudal arcuate nucleus. *Dlx*-1 transcripts in the olfactory epithelium were not reported by these authors, suggesting that the *Dlx*-2 expression domain is more rostral as well. *Dlx*-1 labeling also appears to extend more laterally in the neuroepithelium in some regions. Finally, *Dlx*-2 is not expressed in the mandibular arch mesenchyme as described for *Dlx*-1 (Price et al., 1991).

The most intriguing difference in expression pattern is visualized in frontal sections of the developing diencephalon. Although Price et al. use the classification system of Herrick (1910), which subdivides the diencephalon differently than does that of Altman and Bayer (1986), both genes show the same dorsal limit of expression at the sulcus limitans/sulcus diencephalicus medius, which is the junction of the surface (alar) and basal plates. However, the ventral boundaries of expression are different, such that each gene displays an intriguing and discrete expression domain (termed "segment-like" by Price et al., 1991) along the dorsoventral axis. These data strongly suggest that the *Distal-less* family of homeobox genes may act to specify regional identity in the developing forebrain in a manner analagous to the way the *Hox* genes are believed to act in the hindbrain and neural tube (reviewed in Kessel and Gruss, 1990).

## CONCLUSIONS

We have described a new homeobox gene family in the mouse, which has homology to the *Drosophila Distal-less* gene, and have shown that at least four of its members are expressed during embryogenesis. The *Distal-less* gene family is extremely conserved in the homeodomain motif. There are only seven variable residues among *Distal-less* homeodomain sequences. These are located primarily in the helix 1 and hinge regions, which are known to be among the least conserved elements of the homeodomain (Scott et al., 1989). These variable positions are present in both the *Drosophila Dll* (Cohen et al., 1989) and *Xenopus Distal-less* (Milan Jamrich, personal communication) genes, as well as in all four mouse sequences described here. This extreme conservation strongly suggests that members of the *Distal-less* gene family perform important functions in development.

Using a cDNA probe corresponding to one of the mouse genes, *Dlx*-2, we have demonstrated that this gene is expressed during embryogenesis (from 10.5 days pc to birth) in a region of the developing ventral forebrain (basal telencephalon and ventral diencephalon) and in the ameloblast layer of the tooth anlagen. Although both of the sites of *Dlx*-2 expression are derived from rostral ectoderm of the embryo, their expression may be independently regulated.

The genesis of the vertebrate forebrain is one of the most complicated and poorly understood facets of the development of the CNS. The hindbrain and neural tube develop through inductive interactions with the notochord, which in turn induces the formation of the floor plate (Placzek et al., 1990). The floor plate is a potential source of retinoic acid and is believed to play an important role in establishing cell

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differentiation and polarity in the neural tube (Wagner et al., 1990; Yamada et al., 1991). In contrast, the forebrain is prechordal and develops in the absence of the inductive influences of the notochord and floorplate. This was apparent to early investigators who recognized that the induction and developmental anatomy of these two areas of the CNS were quite distinct (Nieuwkoop, 1952; for a review see Bergquist and Källén, 1954).

In recent years, the molecular basis of the development of the epichordal part of the CNS (the hindbrain and neural tube) has been addressed through the isolation of putative developmental control genes that are differentially expressed in these areas. These include the *Hox* genes, which are expressed in a spatially restricted manner along the neural tube and hindbrain, and the murine *paired* box, or *Pax*, genes, which are expressed along the axis in discrete subregions of the CNS (for a review see Kessel and Gruss, 1989). The most anterior boundary of expression of the *Hox* genes lies in the hindbrain, as is the case for the majority of the *Pax* genes. *En*-1 and *En*-2, vertebrate homolog of the *Drosophila engrailed* gene, are expressed in the developing midbrain (Davis and Joyner, 1988). However, the murine *Distal-less* genes are the first genes found to be expressed exclusively in the forebrain, and this observation suggests that they play a specific role in the development of this region.

It has been shown that the expression boundaries of several members of the *Hox* 2 cluster coincide with segmental neuromeric units (rhombomeres) in the hindbrain (Lumsden and Keynes, 1989; Wilkinson et al., 1989). Neuromeric constrictions can be visualized in the developing prosencephalon, and several different models for the subdivision of this structure have been proposed (Bergquist and Källén, 1954; Keyser, 1972; Puelles et al., 1987). However, it has been shown that neuromeric constrictions in prosencephalon are extremely dynamic and evolve rapidly throughout development, and these observations have raised the question of their relevance. Expression of *Dlx*-2 is not confined to any obvious neuromeric unit, and in fact traverses several, including the boundary between the telencephalon and diencephalon. However, by analogy to the expression of *Hox* genes in more posterior parts of the brain, the *Dlx* expression boundaries may correspond to developmental/neuromeric divisions. Whole-mount in situ hybridization and immunohistochemistry experiments with anti-*Dlx*-2 antibodies may more accurately determine the correspondence of expression domains with neuromeric constrictions.

The expression domains of the *Dlx* genes may define developmental units or compartments in the forebrain, just as the *Hox* genes do in the hindbrain. Thus far, two *Distal-less* genes, *Dlx*-1 (Price et al., 1991) and *Dlx*-2 (this paper), are expressed in overlapping regions in the forebrain. It remains to be seen if other members are expressed there as well.

Our data support the concepts that the basal plate is a distinct developmental entity and that the telencephalic and diencephalic regions of the basal plate are ontogenetically related (discussed in Bergquist and Källén, 1954; Puelles et al., 1987). It is noteworthy that the ventral forebrain in higher mammals, including the olfactory bulb, is derived from a phylogenetically old subdivision of the brain, the paleopallium (Romer, 1976). In this regard, it will be very interesting to see how *Distal-less* homologs are expressed in the brains of lower vertebrates.

The *Dll* gene of *Drosophila* plays an important role in the determination of the proximo-distal axis of the developing limbs (Cohen and Jürgens, 1989). In mouse, *Dlx*-2 transcripts are present in the anterior CNS, and are not detected in limbs. However, there is considerable expression of *Dll* in the developing head of the fly, restricted to the primordia of the antennae and various sense organs, and mutant embryos lack these structures (Cohen and Jürgens, 1989). Thus, the different functions of *Dll* appear to have diverged in vertebrates.

In *Drosophila*, *Dll* is regulated by segmentation genes, and accordingly is located at an intermediate position in the regulatory cascade specifying positional information in the embryo (Cohen and Jürgens, 1990). It is interesting to note that the *Dlx*-2 gene is expressed in the mouse embryo well after formation of the cephalic neural plate, and therefore must also be under the control of other upstream regulatory genes.

**Note Added in Proof**

After this manuscript was submitted, Proteus et al. (Neuron 7:221-229, 1991) reported on the isolation of *Tes*-1,a gene identical to *Dlx*-2.

## MATERIALS AND METHODS

### *Animals*

Inbred mouse strains used for embryological studies were either FVB/N or C57BL/6J. No difference in expression was noted between the strains. Embryos were obtained from superovulated females. Nominal embryonic age was determined by counting noon of the plug day as 0.5 days pc.

### *Preparation of cDNA and Amplification of Homeobox Sequences*

For the isolation of RNA, embryos were homogenized in RNAzol (Cinna-biotex) and processed according to the manufacturer's protocol. cDNA was synthesized from 1 μg RNA in 20 μl of PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$, 0.01% [w/v] gelatin, 200 μM each dNTP) containing 1.6 μg oligodeoxythymidine primer and 200 units of Moloney murine leukemia virus reverse transcriptase (BRL) (Kawasaki, 1990). After incubation for 1 h at

37°C the reaction was inactivated for 10 min at 95°C and 10 μl used for PCR. Homeobox sequences were amplified with degenerate oligonucleotide primers corresponding to aa 3 to 7 (KPRTIY: 5′-GGACTAGTGAARCCNMGNAC-NATHTA-3′) and aa 46 to 51 (KIWFQN: 5′-CGGTCGACG-GRTTYTGRAACCADATYTT-3′) of the *Drosophila Distal-less* homeobox (Cohen et al, 1989) with SpeI and SalI restriction linkers, respectively, at the 5′ ends (IUPAC code: D, not C; H, not G; M, A or C; N, all four; R, A or G; Y, C or T). Amplification (as described in Mackem and Mahon, 1991) consisted of five cycles at an annealing temperature of 45°C with a 2-min ramp to 72°C, followed by 25 step cycles at an annealing temperature of 55°C. Amplified products were purified from a 2% agarose gel, digested with SalI and SpeI, cloned into Bluescript (Stratagene), and sequenced according to the dideoxy method of Sanger et al. (1977).

### *Isolation of cDNA Clones*

An 11.5-day embryonic mouse cDNA library in λgt10 (Clontech) was screened according to standard methods (Sambrook et al., 1990) with a mixture of the 150-bp amplified *Distal-less* homeobox sequences that had been labeled with $^{32}P$ by the random-primer method (Feinberg and Vogelstein, 1983). Hybridization of the filters at 60°C in Church buffer (Church and Gilbert, 1984) was followed by a stringent washing at 60°C in 0.2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7) for 1 h. Inserts were isolated from phage DNA, cloned into Bluescript (Stratagene), and sequenced (Sanger et al., 1977).

### *Northern Blot Analysis*

RNAs were separated on 1.2% formaldehyde-agarose gels (Sambrook et al., 1989) and transferred to Duralon nylon membrane (Stratagene). Blots were hybridized to random-primed $^{32}P$-labeled probes, which had been generated from gel-purified restriction fragments, at 42°C in 50% formamide, 5 × SSC, 1% SDS, 10% dextran sulfate, and 100 μg/ml salmon sperm DNA. Stringent washes were carried out at 65°C in 0.2 × SSC. Fragments used were either the 1.4-kb EcoRI insert from the *Dlx*-2 cDNA clone or the rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA (kindly provided by Sherrie Tafuri).

### *In Situ Hybridization*

Embryos were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Sense and antisense $^{35}S$-labeled riboprobes were generated from appropriately linearized plasmids by in vitro transcription with T3 and T7 RNA polymerases (Stratagene) and [$^{35}S$]UTP under standard conditions. Probes used were derived from plasmids containing either the entire 1.4-kb EcoRI cDNA insert or a 634-bp PstI-EcoRI fragment lacking the homeobox derived from the 3′ end of the cDNA (nucleotides 828-1461). Equivalent results were obtained with both probes. In situ hybridization was performed as previously described (Mackem and Mahon, 1991). The slides were exposed to Kodak NTB-2 emulsion and developed after 10 to 14 days.

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